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Cameron B. Gundersen

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The final technical summary is presented on the accompanying pages:1&2.

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Final Report

The original goal of the proposed experiments was to explore the possibility that mRNA-injected Xenopus oocytes could be used to reconstitute the transmitter release process. The hypothesis was that it might be possible to detect physiologically relevant acetylcholine release from Xenopus oocytes injected first with mRNA from cholinergic neurons and then with synaptic vesicles. For a number of technical reasons that have been discussed in prior reports, this approach has not been successful. In lieu of this effort, our primary focus has shifted toward attempts to clone the cDNA for two presynaptic proteins (the N-type calcium channel and the high affinity choline transporter) and one post-synaptic protein (a glutamate receptor). The status of these efforts is outlined below.

Because of this project's aim of reconstituting ACh release, we were using preparations of mRNA from the uniformly cholinergic neurons of Torpedo electric lobe. These mRNA preparations induce Xenopus oocytes to express functional choline acetyltransferase, an omega-conotoxin-sensitive calcium channel and a partially hemicholinium-3 inhibitable choline transporter. With the intention of trying to clone the cDNA for two of these proteins (the cDNA for choline acetyltransferase has been cloned), we fractionated mRNA on sucrose gradients and found that peaks of mRNA that induce choline uptake were around 2 Kb and that mRNA greater than 6 or 7 Kb induced expression of the calcium channel. In addition we observed that mRNA greater than 6 Kb in mass was predominantly responsible for the responses seen to kainic acid in oocytes injected with total electric lobe mRNA. Thus, in the course of screening size-selected cDNA libraries, we have tested for the presence of kainate receptors as well as calcium channels.

Our cloning strategy has been following several recent precedents in which mRNA is synthesized in vitro from DNA obtained from the cDNA library. This mRNA is injected into Xenopus oocytes which are then tested for the functional expression of the desired protein. The progress we have made for each of the three proteins we are trying to clone is summarized:

Choline transporter: A continuing problem for this project is that the increased uptake of choline in oocytes injected with Torpedo or rat brain mRNA has not been terribly sensitive to inhibition by hemicholinium-3, an agent that fully blocks high affinity choline transport in native tissue at about 1 μM. Although we have performed some functional screening for this transporter using cDNA-library-derived mRNA, another approach potentially may be more useful. A Japanese group has cloned a choline transporter from yeast and we have found labelling of two bands of rat or Torpedo mRNA on Northern blots using the yeast cDNA as a probe. We are currently screening a rat cDNA library using the probe.

Calcium channel: To date we have not seen any evidence of calcium channel expression in oocytes injected with mRNA synthesized off of cDNA templates.

Glutamate receptor - Very recently we have obtained suggestive data that one of our Torpedo cDNA libraries harbors inserts encoding the kainate subclass of glutamate receptor. We are pursuing this lead and expect to know soon whether this library indeed contains the desired clone. If it does, we will proceed immediately to isolate and sequence this clone.

Besides this cloning effort there have been several other projects that have yielded interesting findings concerning the operation of membrane proteins that have been expressed in Xenopus oocytes injected with mRNA from different sources. Summaries are given below of the salient observations in these studies.

1) Barbiturates and human brain Ca channels: Barbiturates at sedative/anesthetic (but not anti-convulsant) concentrations accelerate the inactivation of a Ca channel expressed in Xenopus oocytes injected with human brain RNA. These observations extend previous findings on the mode of operation of these drugs by showing that human brain "N-like" channels are susceptible to barbiturate-depression which may alter nerve cell excitability and neurotransmitter release.

2) Mercuric ion effects on human brain kainate receptors: This investigation revealed that mercuric ions were highly potent, non-competitive antagonists of kainic acid gated channels expressed in oocytes injected with human brain RNA. This observation has functional and structural ramifications for the operation of the kainic acid receptor. Moreover, the results may help to offer insights into the toxicity associated with mercury and its derivatives.

3) Myasthenic syndrome antibodies and their effects on human brain Ca channels: Recent work has implicated the presynaptic Ca channel as a binding site for "auto-antibodies" in the serum of individuals with the myasthenic syndrome. We have tested this possibility using Xenopus oocytes that express human "presynaptic-like" Ca channels and find a depression of Ca currents by these antibodies. We are exploring further the mechanism of this effect.

4) Expression of binding/effector sites for the presynaptic neurotoxin, alpha-latrotoxin, in mRNA-injected Xenopus oocytes. When Xenopus oocytes are injected with rat brain mRNA they acquire binding/effector sites for the spider venom toxin, alpha-latrotoxin. The presence of these sites can be detected electrophysiologically either by monitoring intracellular Ca with Ca-sensitive microelectrodes, or by recording transmembrane currents that are triggered by the toxin-induced rise of cytoplasmic Ca ion activity. These experiments were conducted in collaboration with Dr. A. Grasso of the CNR Laboratory of Cell Biology in Rome as a prelude to anticipated efforts to clone the cDNA for the toxin binding site. However, subsequent studies have suggested that the toxin binding site is composed of at least two distinct polypeptides which makes the expression-cloning effort using oocytes considerably more difficult.